IGF-II/mannose-6-phosphate receptor signaling induced cell hypertrophy and atrial natriuretic peptide/BNP expression via $G\alpha_q$ interaction and protein kinase C- α /CaMKII activation in H9c2 cardiomyoblast cells

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Abstract

The role played by IGF-II in signal transduction through the IGF-II/mannose-6-phosphate receptor (IGF2R) in heart tissue has been poorly understood. In our previous studies, we detected an increased expression of IGF-II and IGF2R in cardiomyocytes that had undergone pathological hypertrophy. We hypothesized that after binding with IGF-II, IGF2R may trigger intracellular signaling cascades involved in the progression of pathologically cardiac hypertrophy. In this study, we used immunohistochemical analysis of the human cardiovascular tissue array to detect expression of IGF2R. In our study of H9c2 cardiomyoblast cell cultures, we used the rhodamine phalloidin staining to measure the cell hypertrophy and western blot to measure the expression of cardiac hypertrophy markers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in cells treated with IGF-II. We found that a significant association between IGF2R overexpression and myocardial infarction. The treatment of

H9c2 cardiomyoblast cells with IGF-II not only induced cell hypertrophy but also increased the protein level of ANP and BNP. Using Leu27IGF-II, an analog of IGF-II which interacts selectively with the IGF2R, to specifically activate IGF2R signaling cascades, we found that binding of Leu27IGF-II to IGF2R led to an increase in the phosphorylation of protein Kinase C (PKC)- α and calcium/calmodulin-dependent protein kinase II (CaMKII) in a $G\alpha_q$ -dependent manner. By the inhibition of PKC- α /CaMKII activity, we found that IGF-II and Leu27IGF-II-induced cell hypertrophy and upregulation of ANP and BNP were significantly suppressed. Taken together, this study provides a new insight into the effects of the IGF2R and its downstream signaling in cardiac hypertrophy. The suppression of IGF2R signaling pathways may be a good strategy to prevent the progression of pathological hypertrophy.

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Introduction

Cardiac hypertrophy can roughly be divided into two types: physiological and pathological (Hunter & Chien 1999). In shorter stresses, physiological hypertrophy is an adaptive response to maintain heart function by increasing the size of cardiomyocytes for strengthening contraction (McMullen *et al.* 2003, Catalucci *et al.* 2008). In contrast, prolonged hypertrophic

stresses may cause non-compensatory pathological hypertrophy that will reactivate the fetal gene expression of atrial natriuretic peptide (ANP) and BNP, and advance to heart failure (Dietz *et al.* 1989, Tissandier *et al.* 1995). However, the transition from physiological hypertrophy to pathological hypertrophy in the molecular mechanisms is unclear. Several extracellular molecules such as insulin-like growth factors (IGFs), angiotensin II (ANGII), and tumour necrosis factor- α have been reported to

be involved in the development of cardiac hypertrophy (Frey *et al.* 2004, Heineke & Molkentin 2006). After selectively binding to receptors on the membrane, these hormones trigger intracellular signaling cascades into the cell nuclei that cause an increase in the expression of the hypertrophic response genes.

IGF-I and IGF-II are members of the IGF family of peptide growth factors. They can bind to two types of cell surface receptors, IGF1R and IGF2R, and have been shown to play a critical role in the development and growth of cells (Jones & Clemmons 1995). IGF1R acts as a receptor tyrosine kinase to trigger a series of mitotic signaling cascades after binding with IGFs (McMullen *et al.* 2004). In contrast, the IGF2R, also known as the 'clearance receptor', stabilizes local IGF concentration through internalization and lysosomal degradation (Boker *et al.* 1997). It has been shown that the binding of IGF-I and IGF-II to the IGF1R induces cell hypertrophy in neonatal ventricular cardiomyocytes (Adachi *et al.* 1994, Miyashita *et al.* 2001). Several investigations further observed that the activation of IGF1R signaling cascade can improve heart contractions and attenuate pathological hypertrophy and fibrosis. However, the increased expression of IGF-II in several animal models with pathological cardiac hypertrophy raised doubts about the role of IGF-II in mediating stressful responses in the heart (Kluge *et al.* 1995, Lee *et al.* 2006).

A few studies have indicated that after binding with IGF-II, IGF2R not only functions in the degradation of IGF-II, but also triggers an intracellular signaling pathway that contributes to the regulation of a variety of physiological functions, such as calcium influx, acetylcholine (ACh) release, and cell migration (Nishimoto *et al.* 1987, McKinnon *et al.* 2001, Hawkes *et al.* 2006). Moreover, the existence of a putative G-protein-binding site within the cytoplasmic domain of the IGF2R suggests that IGF-II may regulate small G proteins that activate signaling pathways through IGF2R (Nishimoto *et al.* 1989, Murayama *et al.* 1990, Okamoto & Nishimoto 1991, Ikezu *et al.* 1995). Based on these findings, we propose that the binding of IGF-II to IGF2R may trigger an intracellular signaling cascade response to cardiac hypertrophy and that the role of this signaling is completely different from that of IGF1R-derived physiological hypertrophy.

In this study, we investigated whether the IGF2R signaling pathway may induce myocardial hypertrophy pathologically by the activation of small G protein and its downstream signaling pathway. We found that the IGF2R was aberrantly expressed in the myocardial infarction tissue. We further found that treatment with IGF-II induced cell hypertrophy in a time-dependent manner and an increase in the protein level of pathological hypertrophy markers ANP and BNP in the H9c2 cardiomyoblast cell. We also found that using Leu27IGF-II, an analog of IGF-II that interacts selectively with the IGF2R (Beukers *et al.* 1991), to specifically activate IGF2R signaling cascades led to an increase in the phosphorylation of protein kinase C (PKC)- α and calcium/calmodulin-dependent protein kinase II (CaMKII) in a G α q-dependent manner. By inhibiting the activity of

PKC- α /CaMKII, the IGF-II-induced cell hypertrophy and upregulation of protein expression of ANP and BNP significantly decreased. Our findings indicated that IGF2R may function as a G-protein-coupled receptor to induce the downstream modulation of PKC- α and CaMKII via the G α q signaling pathway, thereby contributing to the progression of pathological hypertrophy. The suppression of the IGF2R signal may help to prevent the transition from physiological hypertrophy to pathological hypertrophy.

Materials and Methods

Immunohistochemical analysis

The human cardiovascular tissue array (Provitro, Berlin, Germany) was immunostained with an anti-IGF2R antibody (SantaCruz Biotechnology, SantaCruz, CA, USA) using the Ultra Vision LP Detection System (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The tissue array section was dried at 58 °C overnight. Then, this section was deparaffinized in xylene and sequentially hydrated using a graded series of ethanol. The endogenous peroxidase activity was blocked with hydrogen peroxide blocking buffer for 13 min. After rinsing in water for 15 min, the microarray slide was microwave-treated in citrate buffer for 15 min, cooled down to room temperature (RT) for 30 min, and blocked with an UV blocking buffer for 5 min. The primary antibody directed against the peptides 1030–1209 of the rat IGF2R (1:100) was incubated for 30 min. The slide was incubated with the primary antibody enhancing buffer at RT for 20 min. HRP Polymer was added and incubated at RT for 20 min. The IGF2R antibody was located by a universal secondary antibody formulation conjugated to an enzyme-labeled HRP Polymer. After staining with an appropriate substrate/chromogen for 5 min, the slide was counterstained with Harris hematoxylin, dehydrated through a graded series of ethanol to xylene washes, and cover slipped with a permanent mounting medium (Sigma Chemical). The polymer complex was then detected by microscopy (magnification 200 $\times$).

Cell culture

H9c2 cardiomyoblast cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 mM pyruvate in humidified air (5% CO₂) at 37 °C. H9c2 cells were cultured in serum-free medium for 12 h and then treated with or without IGF-I (10⁻⁸ M; Sigma Chemical), IGF-II (10⁻⁸ M; Sigma Chemical), and Leu27IGF-II (10⁻⁸ M; GroPep, Adelaide, Australia). After further incubation for 12 or 24 h, the cells were harvested and extracted for analysis.

Measurement of cell surface area

To measure the cell surface area, cardiomyocytes were stained with rhodamine phalloidin (1:50 dilution) for 20 min to visualize F-actin. Cell images from at least ten randomly chosen fields ($\times 40$ objective) of 60 cardiomyocytes were measured in three separate experiments using NIH image software. Only myocytes that were completely in the field were measured. The effect of IGF-II-mediated increase in cell size was determined.

Protein extraction and western blot analysis

Cultured H9c2 cells were scraped and washed once with PBS. The cell suspension was then spun down, and the cell pellets were lysed for 30 min in lysis buffer (50 mM Tris (pH 7.5), 0.5 M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol, 1 mM basal medium Eagle, 1% Igepal-630, and proteinase inhibitor cocktail tablet (Roche)) and spun down at 12 000 *g* for 10 min. Then, the supernatants were removed and placed into new Eppendorf tubes for western blot analysis. Proteins from the H9c2 cell line were separated on 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific protein binding was blocked in blocking buffer at RT for 1 h (5% milk, 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20). The membranes were blotted with specific antibodies as indicated for each experiment in the blocking buffer at RT for 1 h. Chemiluminescent detection was accomplished with Western Blotting Luminol Reagent (SantaCruz Biotechnology). The densitometric analysis of immunoblots was performed using the AlphaImager 2200 Digital Imaging System (Digital Imaging System, CA, USA).

Antibodies and reagents

The following antibodies were used in this study: anti-ANP (SantaCruz Biotechnology), anti-BNP (SantaCruz Biotechnology), anti-ERK1/2 (SantaCruz Biotechnology), anti-phospho-ERK1/2 (SantaCruz Biotechnology), anti-phospho-PKC- α (Cell Signaling, MA, USA), anti-PKC- α (Cell Signaling), anti-phospho-CaMKII (Cell Signaling), anti-CaMKII (Cell Signaling), anti-G α q (SantaCruz Biotechnology), and anti- α -tubulin (SantaCruz Biotechnology). All of the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) and the enhanced chemiluminescence kit were purchased from Santa Cruz Biotechnology. IGF-I and IGF-II were purchased from Sigma, and Leu27IGF-II was obtained from GroPep. The calcineurin inhibitor FK506 and cyclosporin A were purchased from Calbiochem (CA, USA) and Sigma respectively. PKC- α /CaMKII inhibitor cocktail was obtained from Upstate. SB203580 (p38 MAP kinase inhibitor; Promega), U0126 (MEK1 and MEK2 inhibitor) and SP600125 (JNK inhibitor) were purchased from Promega.

siRNA and transfection

Double-stranded small interfering RNA (siRNA) sequences targeting guanine nucleotide-binding protein, α q polypeptide (G α q; GenBank accession number NM_031036), mRNAs were obtained from Dharmacon (Lafayette, CO, USA). A nonspecific duplex (5'-CAGUGGAGAUCAACGUGC AAGUU-3'; Dharmacon), which did not significantly affect G α q mRNA and protein levels relative to the untransfected controls, was used as a control. The concentration of siRNA and the time of incubation were tested. H9c2 cardiomyoblast cells were plated in 100 mm well plates in DMEM without fetal bovine serum and transfected with double-stranded siRNA using the DharmaFECT Duo Transfection Reagent (Dharmacon) according to the manufacturer's instructions. To assess gene silencing, G α q protein levels were detected by immunoblotting.

Statistical analysis

The relative intensities of protein were analyzed using the Digital Sciences 1D program from Kodak Scientific Imaging Systems. All of the results were expressed as means $\pm$ s.d., or as the means and the coefficient of variation of three to five separate experiments, as indicated. The transfection experiments were performed in triplicate. Standard curves were run, and the data that were obtained fell within the linear range of the curve. In addition, all values were normalized to their respective lane loading controls. The densitometric analysis of immunoblots in bar figures (Figs 3f, 4b, d, 5b and d) was performed using one-way ANOVA with preplanned contrast comparisons against the control group (serum-free) or against the Leu27IGF-II group. Results in Figs 2b, d, 3b and d were analyzed by unpaired Student's *t*-test. In all cases, $P < 0.05$ was considered significant.

Results

Expression analysis of IGF2R in myocardial infarction tissues

To examine the correlation between the expression levels of IGF2R protein and myocardial infarction, we performed immunohistochemical analysis of human cardiovascular tissue array containing 10 normal heart and 28 infarcted myocardium tissues. Representative images demonstrating positive or negative infarcted myocardium staining compared with normal human heart tissue are shown in Fig. 1. A total of 25 (65.7%) showed positive staining for IGF2R. Thus, 13 (34.3%) could be categorized as absent or minimal expression for IGF2R. Out of the infarcted myocardium samples, 18 (64%) showed a strong expression of IGF2R and 7 (25%) showed moderate expression. The remainder appeared to have no more staining than the normal heart tissue. None of the ten normal tissue samples on the slide showed any IGF2R overexpression. Only 3 out of the 28 infarcted myocardium samples showed staining beyond

Figure 1 Detection of IGF2R expression in human cardiovascular tissue array by immunohistochemistry (IHC). N1 shows the antibody staining weakly in a normal heart section. I1–I5 show the infarcted myocardium staining strongly with the anti-IGF2R antibody. Magnification $\times 200$.

background levels and none had strong staining. We found a significant association between IGF2R overexpression (scored as moderate/strong) and myocardial infarction. Overall, then, 25 out of the 28 infarcted myocardium samples (89%) examined by immunohistochemistry showed a significant overexpression of IGF2R.

IGF-II induction of H9c2 cardiomyoblast cell hypertrophy and reactivation of ANP and BNP expression

We investigated whether the treatment with IGF-II would directly induce cell hypertrophy and reactivate the expression of hypertrophy markers ANP and BNP in H9c2 cardiomyoblast cells, and compared its effect with IGF-I. The rhodamine phalloidin stain revealed, when compared with untreated controls, that there was 1.8- and 2.3-fold increase in cell surface size in the cells treated with IGF-I and IGF-II respectively (Fig. 2a and b) for 24 h. However, the treatment with IGF-II, but not with IGF-I, for 12 h significantly elevated the cell surface size (Fig. 2a and b) suggested that IGF-I and IGF-II induced cell hypertrophy probably by triggering a distinct signal transduction. Furthermore, western blot revealed that there was a sixfold increase in the protein levels of ANP and BNP in cells treated with IGF-II (Fig. 2c and d) but not in cells treated with IGF-I. Although IGF-I

and IGF-II both induced cell hypertrophy (Fig. 2a and b), the increased ANP and BNP levels were detected only in the cells treated with IGF-II, indicating that IGF2R plays a crucial role in the induction of ANP and BNP by activating the intracellular signaling pathway involved in pathological hypertrophy (Dietz *et al.* 1989, Tissandier *et al.* 1995).

Leu27IGF-II activation of the PKC- α /CaMKII signaling through G α_q

Using Leu27IGF-II to exclude other effects derived from insulin and IGF1Rs in H9c2 cardiomyoblasts (Beukers *et al.* 1991), we attempted to clarify whether the IGF-II-induced small G-protein-sensitive signaling pathway is mediated by IGF2R. Western blots revealed that treatment with IGF-I and IGF-II, but not with Leu27IGF-II, increased the level of ERK1/2 phosphorylation at 30 min, (Fig. 3a and b), suggesting that Leu27IGF-II did not activate the IGF1R downstream effectors. We also observed that treatment with IGF-II and Leu27IGF-II both resulted in a significant increase in the phosphorylation of PKC- α and CaMKII (Fig. 3c and d), suggesting that the effects might occur through IGF2R. When compared with IGF-II, Leu27IGF-II had stronger and faster effects on the phosphorylation of PKC- α and CaMKII (Fig. 3c and d), which might be due to the fact

Figure 2 IGF-II induction of cell hypertrophy in H9c2 cardiomyoblast cells. (a and b) After treatment with IGF-I (10^{-8} M) and IGF-II (10^{-8} M), H9c2 cardiomyoblasts were stained with rhodamine phalloidin for F-actin to detect the cell size. DAPI staining was used to mark nuclei. The results showed that IGF-I and IGF-II increase of ~ 2.1 - and 3-fold in cell surface size respectively, when compared with untreated control. The cell surface area of at least 50 myocytes from 10 randomly selected fields in three separate experiments (the number of replications (n value) is three to five for each groups) was measured by a computed image analyzer; data are presented as a percentage compared with untreated control (mean $\pm$ S.E.M). Bars indicate averages. Statistical significance: * $P < 0.05$; # $P < 0.01$, IGF-I or IGF-II versus untreated controls. (c and d) Western blot of protein lysates from H9c2 cardiomyoblasts treated with IGF-I (10^{-8} M) and IGF-II (10^{-8} M) at 12 and 24 h using anti-ANP antibody and anti-BNP antibody respectively as the cardiac hypertrophy marker. Protein levels of ANP and BNP increased in the presence of IGF-II, but not in cells treated with IGF-I. The blots were measured by densitometry. Data are presented as means $\pm$ S.E.M. Bars indicate averages, * $P < 0.05$. $n =$ three independent experiments for each data point.

that Leu27IGF-II has a higher affinity to bind with IGF2R than with IGF-II. We further investigated whether $G\alpha_q$ might be involved in the Leu27IGF-II-induced phosphorylation of PKC- α and CaMKII, used guanine nucleotide-binding protein, alpha q polypeptide ($G\alpha_q$), siRNA to

disrupt the expression of $G\alpha_q$ protein in H9c2 cardiomyoblast cells. As shown in Fig. 3e and f, we found a significantly greater reduction in the phosphorylation of PKC- α and CaMKII in cells transfected with $G\alpha_q$ siRNA than in cells treated with Leu27IGF-II alone, implying that the siRNA-mediated

Figure 3 $G\alpha q$ is necessary for the activation of PKC- α and CaMKII by IGF-II and Leu27IGF-II. (a and b) Western blot analysis of H9c2 cardiomyoblasts treated with IGF-I (10^{-8} M), IGF-II (10^{-8} M) or Leu27IGF-II (10^{-8} M) respectively at 30 and 60 min using anti-phospho-ERK1/2 antibody, anti-ERK antibody and anti- α -tubulin antibody. Phospho-ERK1/2 protein levels increased with IGF-I or IGF-II treatment, but not with Leu27IGF-II treatment. These blots were quantified by densitometry. α -Tubulin served as a loading control. Data are presented as means $\pm$ S.E.M. Bars indicate averages, $*P < 0.05$. $n = 3$ independent experiments for each data point. (c and d) Western blot analysis of H9c2 cardiomyoblasts treated with IGF-II (10^{-8} M) and Leu27IGF-II (10^{-8} M) using indicated antibodies. There was an increase in PKC- α and CaMKII phosphorylation in the treatment with both IGF-II and Leu27IGF-II. Results are from four independent experiments run in triplicate on cultured cells. Data were quantified by densitometry and are presented as means $\pm$ S.E.M. Bars indicate averages. Statistical significance: $*P < 0.05$; $\#P < 0.01$, IGF-II or Leu27IGF-II versus untreated controls. $n = 3$ independent experiments for each data point. (e and f) H9c2 cardiomyoblasts were transiently transfected with mock or $G\alpha q$ siRNA for 24 h and either untreated or stimulated with Leu27IGF-II (10^{-8} M) for an additional 48 h, after which the protein lysates were prepared from those cells. Depletion in $G\alpha q$ protein was seen only in $G\alpha q$ siRNA-transfected cells by immunoblotting. There was a significant reduction in the phosphorylation of PKC- α and CaMKII in transfected $G\alpha q$ -directed shRNA, compared with cells treated only with Leu27IGF-II, as detected by immunoblotting. These results suggest that the shRNA-mediated knockdown of $G\alpha q$ inhibits Leu27IGF-II-induced PKC- α and CaMKII activation. α -Tubulin served as a loading control. Data were quantified by densitometry and are presented as means $\pm$ S.E.M. Bars indicate averages. $*P < 0.05$ values were based on comparison with untreated controls; $\#P < 0.05$ values were based on comparisons with cells treated with Leu27IGF-II. $n = 3$ independent experiments for each data point.

knockdown of $G\alpha q$ inhibits Leu27IGF-II-induced PKC- α and CaMKII activation. Taken together, these findings indicate that, in response to the ligand, IGF2R may act as a GPCR to modulate $G\alpha q$ and activate its downstream effectors.

Inhibition of PKC- α /CaMKII signaling blocks IGF-II and Leu27IGF-II-induced cell hypertrophy and upregulating ANP/BNP

In order to find out whether the activation of PKC- α and CaMKII is required for IGF-II and Leu27IGF-II to induce cell hypertrophy and upregulate ANP and BNP, we used a variety of protein kinase inhibitors to suppress intracellular

signaling cascades in H9c2 cardiomyoblast cells exposed to IGF-II or Leu27IGF-II. We found that the inhibition of either PKC- α /CaMKII or ERK1/2 activities significantly prevented IGF-II-induced cell hypertrophy (Fig. 4a and b). The results suggest that IGF-II induced cell hypertrophy via two distinct pathways. However, the Leu27IGF-II-induced cell hypertrophy was rescued by inhibiting the PKC- α /CaMKII activity (Fig. 5a and b), indicating that the binding of Leu27IGF-II to IGF2R induced cell hypertrophy by activating the PKC- α /CaMKII signaling pathway, but not the ERK1/2 pathway. We further found that once PKC- α /CaMKII activity was inhibited, both IGF-II and Leu27IGF-II induction of ANP and BNP protein expression could be

Figure 4 IGF-II induction of cell hypertrophy through ERK1/2 and PKC- α signaling pathways in H9c2 cardiomyoblasts. (a and b) After preincubation with several protein kinase inhibitors, as indicated, for 2 h, H9c2 cardiomyoblasts were treated with IGF-II (10^{-8} M) for 24 h and stained with phalloidin-rhodamine. DAPI staining was used to mark nuclei. There was a reduction in IGF-II-induced cell hypertrophy in the presence of either U0126 (ERK1/2 inhibitors) or PKC- α /CaMKII inhibitor, suggesting that IGF-II induced cardiomyocyte hypertrophy by activating ERK1/2 and PKC- α /CaMKII. The cell surface area of at least 50 myocytes from 10 randomly selected fields in three separate experiments was measured by a computed image analyzer; data are presented as a percentage compared with no IGF-II treatment (mean $\pm$ s.e.m.). ** $P < 0.05$ compared with control; * $P < 0.05$ compared with IGF-II stimulation. $n =$ three to five independent experiments for each data point. (c and d) The extraction of protein lysates from H9c2 cardiomyoblasts treated with IGF-II (10^{-8} M) after being exposed to several protein kinase inhibitors for 2 h, as indicated. Immunoblotting experiments were performed to detect the protein levels of ANP and BNP as cardiac hypertrophy markers. Anti-ANP and anti-BNP antibodies were used to reveal the diminution of IGF-II-dependent ANP and BNP expression in the presence of either U0126 (ERK1/2 inhibitors) or PKC- α /CaMKII inhibitors. Data were quantified by densitometry and are presented as means $\pm$ s.e.m. Bars indicate averages. * $P < 0.05$ values are based on comparison with untreated controls; # $P < 0.05$ values were based on comparisons with cells treated with IGF-II. $n =$ three independent experiments for each data point.

reduced (Figs 4c and d, 5c and d). Taken together, these findings demonstrate that IGF2R signaling induced cell hypertrophy and upregulated ANP and BNP by activating PKC- α /CaMKII, whereas IGF1R signaling only induced cell hypertrophy, but did not regulate ANP and BNP, via ERK1/2 activation.

Discussion

In this study, we investigated whether the IGF2R signaling pathway is involved in cardiac hypertrophy and if so, wanted to know whether it occurred through the regulation of the small G-protein-derived signaling pathway. These findings suggest that, by modulating G α_q and the activation of its downstream effectors PKC- α /CaMKII, the IGF2R signaling

pathway may contribute to the progression of pathological hypertrophy. A depiction of this process is shown in Fig. 6.

Although IGF-I and IGF-II have highly homologous protein structures and play similar roles in cell growth and development, they have been reported to act differently in the regulation of endogenous ACh release in hippocampal formation and promotion of fetal growth (Kar *et al.* 1997, Sferruzzi-Perri *et al.* 2006). In the heart, IGF-II is thought to be a potential candidate for the treatment of heart failure via the IGF1R signaling pathway (Delafontaine & Brink 2000, Climent *et al.* 2007). Previous investigations have reported that when the IGF1R-dependent pathway is blocked, IGF-II induces hypertrophy of cardiomyocytes via an IGF2R-dependent pathway (Huang *et al.* 2002). In the present study, we further found that treatment with both IGF-I and IGF-II induced cell hypertrophy, but with only IGF-II induced the protein level increase of ANP and BNP,

Figure 5 Inhibition of PKC- α /CaMKII signaling blocks Leu27IGF-II-induced cell hypertrophy and upregulation of ANP/BNP. (a and b) After preincubation with several protein kinase inhibitors, as indicated, for 2 h, H9c2 cardiomyoblasts were treated with Leu27IGF-II (10^{-8} M) for 24 h and stained with phalloidin-rhodamine. DAPI staining was used to mark nuclei. There was a reduction in Leu27IGF-II-induced cell hypertrophy in the presence of PKC- α /CaMKII inhibitor, suggesting that Leu27IGF-II induced cardiomyocyte hypertrophy by activating PKC- α /CaMKII. The cell surface area of at least 50 myocytes from 10 randomly selected fields in three separate experiments was measured by a computed image analyzer; data are presented as a percentage compared with no IGF-II treatment (mean $\pm$ S.E.M.). ** $P < 0.05$ compared with control; * $P < 0.05$ compared with Leu27IGF-II stimulation. $n =$ three to five independent experiments for each data point. (c and d) The extraction of protein lysates from H9c2 cardiomyoblasts treated with Leu27IGF-II (10^{-8} M) after being exposed to several protein kinase inhibitors for 2 h, as indicated. Immunoblotting experiments were performed to detect the protein levels of ANP and BNP, as cardiac hypertrophy markers. Anti-ANP and anti-BNP antibodies were used to reveal the reduction of Leu27IGF-II-dependent ANP and BNP expression in the presence of PKC- α /CaMKII inhibitors. Data were quantified by densitometry and are presented as means $\pm$ S.E.M. Bars indicate averages. * $P < 0.05$ values are based on comparison with untreated controls; # $P < 0.05$ values are based on comparisons with cells treated with Leu27IGF-II. $n =$ three independent experiments for each data point.

which act as pathological hypertrophy markers (Fig. 2). We also found that a positively significant association between overexpressed IGF2R protein and infarcted myocardium (Fig. 1). These results indicated that IGF-II might induce pathological cell hypertrophy via IGF2R and is different from IGF-I-induced physiological hypertrophy.

The physiological functions of IGFs mediated by three plasma membrane receptors (Jones & Clemmons 1995), including the IGF-I, IGF-II, and insulin receptors, have made it difficult to identify the specific role that IGF2R plays in the mediation of a given biological response. We used the Leu27IGF-II analog, which interacts selectively with the IGF2R (Beukers *et al.* 1991), to overcome the cross talk among these receptors, providing us an opportunity to reexamine the role of IGF2R in cell signaling. We found that treatment with either IGF-II or Leu27IGF-II led to an increase in the phosphorylation of PKC- α and CaMKII

(Fig. 3). Studies have found that the cross-talk between IGF2R signals and the small G-protein $G\alpha i$ regulates cell behavior by activating specific intracellular signaling cascades in a pertussis toxin-sensitive manner (Nishimoto *et al.* 1987, McKinnon *et al.* 2001, Hawkes *et al.* 2006). Our data indicated that Leu27IGF-II-induced the increment of phospho-PKC- α and phospho-CaMKII is $G\alpha q$ required (Fig. 3). It would be interesting to further investigate how IGF2R cooperates with $G\alpha q$ to activate its downstream signaling cascades.

In this study, we demonstrated that there are two signaling pathways, ERK1/2 and PKC- α /CaMKII, involved in IGF-II-induced cell hypertrophy, and that only PKC- α /CaMKII activation is needed for IGF-II-increased expression of ANP and BNP (Fig. 4a–d). Our findings showed that Leu27IGF-II enhanced the phosphorylation of PKC- α and CaMKII, but did not have the same effect on ERK1/2 phosphorylation (Fig. 3),

Figure 6 A model for the IGF-II signaling pathway in cardiac hypertrophy. In this study, we observed that IGF-II induced cardiac hypertrophy via at least two signaling pathways: one signaling pathway is ERK1/2, which has downstream effects on the IGF1R and the other is PKC- α /CaMKII, which is dependent on the IGF2R. The Leu27IGF-II analog was used to ensure that activation of the IGF2R would increase the phosphorylation of PKC- α and CaMKII. RNAi disruption of G α q significantly depressed the level of Leu27IGF-II-induced PKC- α /CaMKII phosphorylation in H9c2 cardiomyoblasts cell. The inhibition of either PKC- α /CaMKII or ERK1/2 activation significantly prevented IGF-II-induced cell hypertrophy, but once PKC- α /CaMKII activation was inhibited, IGF-II induction of ANP and BNP protein expression could be prohibited. We hypothesize that after binding with IGF-II, the IGF2R may function as a G-protein-coupled receptor that can trigger intracellular PKC- α /CaMKII signaling cascades contributing to pathological hypertrophy, a mechanism that differs from that responsible for IGF1R-derived physiological hypertrophy.

suggesting that binding of IGF-II to IGF2R could induce cell hypertrophy and induction of ANP and BNP. Moreover, specifically activated IGF2R signaling by Leu27IGF-II triggered the PKC- α /CaMKII signaling, in order to induce cell hypertrophy, and upregulated ANP and BNP (Fig. 5), which confirm the results in Figs 3 and 4 respectively. For further research, it would be interesting to investigate whether IGF2R, in addition to serving as 'clearance receptor' for IGF-II, is involved in the regulation of various physiological functions including cell metabolism, development and growth, all of which are modulated by IGF-II.

Numerous studies have implicated that the activation of PKC- α and CaMKII signaling in response to calcium influx (Molkentin 2006, Ferrero *et al.* 2007) might play a critical role in the G α q-induced pathological cardiac hypertrophy, cardiac contractile failure, and apoptosis of cardiomyocytes (D'Angelo *et al.* 1997, Adams *et al.* 1998, Mende *et al.* 1998, Wettchurek *et al.* 2001, Braz *et al.* 2004). It is possible that by activating PKC- α and CaMKII, IGF2R signaling may be involved in the regulation of pathological cardiac remodeling and the progression from adaptive cardiac hypertrophy to cardiac failure. The study of BALB/c 3T3 cells shows that IGF-II stimulates the calcium influx involved with GTP-binding protein (Nishimoto *et al.* 1987). Based on our results,

we propose that after IGF-II-binding, the IGF2R changes conformation, allowing G α q to bind and activate phospholipase C (PLC)- β in the cardiomyocytes (Rockman *et al.* 2002). This results in a change in the influx of intracellular calcium via inositol trisphosphate and contributes to the activation of calcium-dependent protein kinase PKC- α and CaMKII (Rockman *et al.* 2002).

In conclusion, the results of this study suggest that an overactivation of the IGF2R pathway might cause pathological hypertrophy by modulating G α q and the activity of its downstream effectors PKC- α /CaMKII to induce ANP and BNP expression. These new insights might be used to prevent the transition from physiological hypertrophy to the pathological hypertrophy in the heart.

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